

## Expression and secretion of recombinant actinidin (cysteine protease) in *Saccharomyces cerevisiae*

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### Abstract

Two variants of actinidin-encoding DNA sequences have been constructed and expressed in yeast *Saccharomyces cerevisiae*. The two variants represented different regions of full-length actinidin cDNA. The first variant (R2) consisted of mature actinidin sequences (starting from amino acid number 127) plus the C-terminal extension up to amino acids number 384. The second variant (R5) consisted of the N-terminal extension, starting from amino acid number 34, spanned through the mature actinidin sequences, plus the whole C-terminal extension (amino acid number 389). It was observed that, upon expression in *S. cerevisiae* using the yeast expression-secretion vector, only the R2 variant resulted in the secreted protein reacted immunogenically with anti-actinidin antibody. The secreted protein, however, had a lower molecular weight as compared to the native actinidin, suggesting that the precursor actinidin was not correctly processed. Nevertheless, it was demonstrated that removal of specific amino acid motifs within the actinidin precursor had resulted in secretion of actinidin into the culture supernatant.

*Keywords:* actinidin, *Saccharomyces cerevisiae*, protein translation, protein secretion

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### Introduction

Yeast *Saccharomyces cerevisiae* has been widely used for expression of many recombinant proteins. It has the ability to express and secrete foreign proteins which gives an advantage for industry as secretion will ease the purification of the protein. Actinidin is a cysteine protease found in the fruit of Chinese gooseberry (*Actinidia chinensis*) or Kiwi fruit, constituting a polypeptide chain of 220 residues, having an Mr of 23.6 kDa (Carne and Moore, 1978; Baker, 1980). It is grouped in the class of plant thiol proteinases which includes papain,

ficin and stem bromelain (McDowall, 1970; Baker, 1980). It resembles papain in that it catalyses the hydrolysis of benzoyl-L-arginine ethyl ester, has a broad range of optimum pH from 5 - 7 as well as substrate specificity (Johnson *et al.*, 1987).

The cDNA as well as the genomic DNA sequences of actinidin have been reported (Praekelt, 1987; Praekelt *et al.*, 1988; Podivinsky *et al.*, 1989; Snowden and Gardner, 1990). Full-length actinidin-encoding DNA contains an ORF of 380 codons, encoding mature actinidin sequences (220 amino acids) flanked by N and C-terminal extensions of 126 and 33 amino acids, respectively.

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The mature actinidin protein was processed from a precursor polypeptide and has the size of 23.6 kDa (Praekelt, 1987; Praekelt *et al.*, 1988). The first 20 amino acids of actinidin show hydrophobic characters typical of a signal sequence, suggesting that the protein enters the secretory pathway.

Several variants of actinidin-encoding DNA sequences have been successfully expressed in *Saccharomyces cerevisiae* (Yuwono, 1991). The protein, however, was found intracellularly despite the fact that the expression was carried out using an expression-secretion vector. It was also observed that the protein expressed was incompletely processed when the amino-terminal extension-coding DNA sequence was removed. On the other hand, the full-length actinidin cDNA, was expressed intracellularly and processed in a protease-proficient yeast strain, suggesting that both amino- and carboxy-terminal extensions were required for correct processing of the protein. Similarly, when actinidin was expressed in tobacco, it was also demonstrated that the carboxy-terminal extension was required for its stability (Paul *et al.*, 1995).

It was also observed that the actinidin precursor sequence contains amino acid motifs, i.e. QR(TN) and QR at its N-terminal extension region and C-terminal extension region, respectively. Those motifs were putatively assumed as having an important role in directing the protein into intracellular organel, presumably vacuole (Yuwono, 1991). Similar motifs have also been found in several proteins destined to vacuole, such as yeast carboxypeptidase Y (QRPL) [Johnson *et al.*, 1987; Valls *et al.*, 1987], phaseolin (QRFD), pea legumin (QRDE), ricin (QRDG), and phytohemagglutinin (QRDA) [Tague *et al.*, 1990]. Therefore, it was of interest to examine whether the removal of such motif from actinidin protein

sequence would divert the protein into extracellular space.

This paper describes the expression of two different variants of recombinant actinidin gene constructions represented different regions of full-length actinidin cDNA. The first variant (R2) consisted of mature actinidin sequences (starting from amino acid number 127) plus the C-terminal extension up to amino acids number 384. The second variant (R5) consisted of the N-terminal extension, starting from amino acid number 34, spanned through the mature actinidin sequences, plus the whole C-terminal extension (amino acid number 389). The two variants were expressed using yeast expression-secretion vector by employing the leader sequence of *MF $\alpha$ 1* gene of *S. cerevisiae* to express and drive the protein to the secretion pathway.

## Materials and methods

### Microorganism

Bacteria *Escherichia coli* DH5 $\alpha$  [F', f80dlacZDM15, *recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17*(r<sub>K</sub><sup>-</sup> m<sub>K</sub><sup>+</sup>), *supE44*, *relA1*, *deoR*, D(lacZYA-argF) U169], was used as the host for cloning and preparation of recombinant DNA.

Yeast *S. cerevisiae* DBY746 (MAT $\alpha$ his3-D1 *leu2-3,112 ura3-52 trp1-289*) was used as the host for the expression of actinidin-encoding gene.

### Plasmid

Plasmid used for the expression of the actinidin-encoding gene was pYSV9 (kindly supplied by Dr. Alan Mileham through Dr. Peter A. Meacock, Department of Genetics, University of Leicester, UK). This plasmid is an expression-secretion vector designed for *S. cerevisiae* system. It contains a leader

sequence of *MF $\alpha$ 1* required for regulating the expression of the cloned gene and directing the product of expression into secretion pathway.

### PCR amplification of actinidin-encoding DNA

Two variants of actinidin-encoding gene were created by PCR amplification targeted to a specific region using pEMBL-KIWI which carried the full-length actinidin cDNA (Yuwono, 1991) as the DNA template. Primers used for amplification were as follows: (1) primer TB2 : 5'-GG GAA GCT T TG CCG AGT TAT GTT GAT-3' and primer P8 : 3'-CCT CAT CTG CTA CCT ATT CAG CTG TTT-5' (for variant R2), and (2) primer P7 : 5'-GAA GCT TTG ACA AAC GAT GAA GTC AAG-3' and primer TW3 : 3'-GTC TCC ATG TCG CGA ATT CAG CTG TTT-5' (for variant R5).

Restriction sites were added in the design of primers to facilitate cloning procedures, i.e.: *Hind*III site in primers TB2 and P7, and *Sal*I site in primers P8 dan TW3 (bold and italic letters).

Variant of actinidin-encoding DNA R2 was created by PCR amplification in a total volume of 50 ml with a composition of: 5 ml 10X buffer PCR, 1 ml 10mM dNTP, 2 ml primer TB2 (255 ng/ml), 4 ml primer P8 (109 ng/ml), 2 ml DNA template (plasmid pKIWI-450, 2.1 mg/ml), 5 ml *Taq* DNA polymerase (1U/ml), 31 ml dH<sub>2</sub>O.

Variant of actinidin-encoding DNA R5 was created by PCR amplification in a total volume of 50 ml with a composition of: 5 ml 10X buffer PCR, 1 ml 10mM dNTP, 5 ml primer P7 (92 ng/ml), 2 ml primer TW3 (250 ng/ml), 2 ml DNA template (plasmid pKIWI-450, 2.1 mg/ml), 5 ml *Taq* DNA polymerase (1U/ml), 30 ml dH<sub>2</sub>O.

PCR was performed under the following condition: initial denaturation at 95°C for 5 min, followed by primer annealing at 55°C

for 5 min and continued with 25 cycles of polymerisation at 72°C for 1 min 48 sec, denaturation at 95°C for 1 min 36 sec, and primer annealing at 55°C for 1 min 48 sec. At the end of cycles, an extension of polymerisation was performed at 72°C for 5 min.

Following amplification, the PCR fragments were purified by using *DNA Clean-up Kit* (Promega). Afterwards, the PCR fragments were digested with *Hind* III dan *Sal* I and electrophoresed on an agarose gel. The digested PCR fragments were then extracted from gel by using *Agarose Gel DNA Extraction Kit* (Boehringer Mannheim). The extracted fragments were then ligated with the expression and secretion plasmid.

### DNA manipulation and transformation

PCR-amplified actinidin DNA variants were subsequently cloned into the *Hind*III-*Sal*I sites of the pYSV9 vector to create an in-frame fusion with the yeast  $\alpha$ -factor leader sequence present in the vector. The recombinant plasmids were then used to transform *Escherichia coli* DH5 $\alpha$  using the heat-shock method. All cloning procedures were essentially as described by Sambrook *et al.* (1989). The recombinant actinidin gene constructions, designated as pYSV-R2 and pYSV9-R5, were then used to transform yeast *S. cerevisiae* strain DBY 746 using lithium acetate-induced transformation method (Rose *et al.*, 1990).

### Yeast cultivation

*S. cerevisiae* carrying recombinant actinidin gene constructions were cultivated in broth medium of Yeast Extract Peptone Dextrose (YEPD) (10 g/l yeast extract, 20 g/l peptone, 2% glucose). The culture was incubated at 30°C for 16 h with shaking. Afterwards, the cells were pelleted by centrifugation and the supernatant was analysed for the presence of secreted recombinant actinidin.

The analysis of recombinant actinidin expression was carried out by cultivating the yeast in 200 ml medium. Culture supernatant was subjected to column chromatography using DEAE Sephacel matrix equilibrated with 100 mM KCl and 100 mM potassium phosphate buffer to reach pH value of 6.5. Chromatography was performed using 40 ml volume of matrix in a glass column of 1.0 cm diameter. Supernatant was flown into the matrix sequentially then eluted using a gradient of 0.1 M KCl–1.0 M KCl in 100 mM phosphate buffer pH 6.5. A total of 70 fractions were collected from the column followed by spectrophotometric analysis at  $A_{280}$  to obtain an estimate of its concentration. Peaks of protein collected were pooled for subsequent immunoblotting analysis.

As a control, yeast culture carrying the vector DNA only (pYSV9), without any actinidin-encoding DNA inserted, was also cultivated in YEPD and chromatographed similarly as described above.

### Immunoblotting analysis

Selected peaks of protein were electrophoresed on an SDS-PAGE (Hames and Rickwood, 1990) followed by blotting on to a nitrocellulose membrane. Subsequently, proteins on the membrane were detected by using immunoblotting technique. The first antibody used (at 1 : 250 dilution) was a polyclonal antibody raised in chicken against purified actinidin extracted from Kiwi fruit. Detection of the antigen was carried out by using anti-chicken IgG alkaline phosphatase conjugate as the second antibody (at 1 : 1000 dilution) followed by colour development using nitroblue tetrazolium and bromocresol indolyl phosphate. All immunoblotting protocols were essentially as described by Yuwono (1991).

## Results and Discussion

### PCR amplification of actinidin-encoding DNA sequences

Variants of actinidin-encoding DNA sequences were obtained by selective amplification of full-length actinidin cDNA (Fig. 1). Variant R2 spanned from mature actinidin sequence (amino acid no. 127 from the initiating methionine codon) to amino acid no. 384, which includes part of the C-terminal extension, just before the QR motif. This variant, therefore, does not include any QR motif. Variant R5, on the other hand, spanned from amino acid no. 34 (which lies within the N-terminal extension, just after the QR motif) to amino acid no. 389 which is the last amino acid within the C-terminal extension before the STOP codon. Variant R5 includes almost all the N-terminal extension region (excluding the QR motif) and the whole C-terminal extension. Therefore, variant R5 includes only the QR motif within the C-terminal extension.

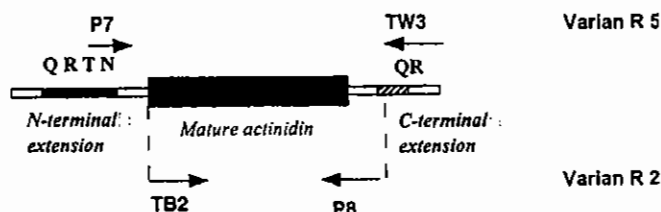


Figure 1. Scheme of PCR amplification of full-length actinidin cDNA to obtain variant R2 and R5. QR(TN) is the putative vacuolar targeting signal. Mature actinidin is the region of actinidin-encoding DNA sequence which results in the mature actinidin protein, while the N-terminal extension and C-terminal extension will be proteolytically processed.

### Cloning of actinidin-encoding DNA variants

Variants of actinidin-encoding DNA R2 and R5 were then cloned into a yeast expression-secretion vector, pYSV9, after digesting it with *Hind*III and *Sal*I. Cloning of the actinidin-encoding DNA variants into *Hind*III site of pYSV9 creates an in-frame fusion with the  $\alpha$ -factor pre-pro leader

sequence present in the vector. The  $\alpha$ -factor is a pheromone used for mating which is naturally secreted out of the cell. The *Hind*III site is actually part of the sequence encoding (Glu-Ala)<sub>2</sub> amino acids of the  $\alpha$ -factor pre-pro leader sequence. The (Glu-Ala) sequence is a part of the pro- $\alpha$ -factor which will be processed by dipeptidyl aminopeptidase A (DPAPase A) during the processing of the  $\alpha$ -factor. Such in-frame fusion will direct the expression of the inserted gene using regulatory region of *MF $\alpha$ 1* gene. The expressed polypeptide will then be directed toward the secretion pathway using the leader sequence of the *MF $\alpha$ 1* gene. Following its transfer to the secretion pathway, the heterologous polypeptide will then be processed using the  $\alpha$ -factor processing system. The heterologous polypeptide will be cleaved from the  $\alpha$ -factor pro-region by an endoprotease encoded by *KEX2* gene. Afterwards, the (Glu-Ala) sequence will be cleaved by DPAPase A encoded by *STE13* gene (Brake, 1989).

The R5 variant was created by PCR amplification using specifically designed primers that contained *Hind*III site at its 5' end. However, a new amino acid sequence (TTG/leucine) had to be inserted in a way to prevent an out-of-frame fusion with the  $\alpha$ -factor pre-pro-leader sequence. Therefore, the sequences of the R5 variant at the junction of fusion with  $\alpha$ -factor leader sequence is as follows:

*MF $\alpha$ 1* leader sequence-GAAGCTTTG ACA AAC..3'  
*Hind*III leu thr asn

Threonine and asparagine are the amino acids number 34 and 35, respectively, of the N-terminal extension of actinidin, while leucine is the added amino acid sequence.

In the design of primers for amplification of R2 variant, on the other hand, no additional amino acid sequence was added.

Therefore, the sequences at the junction between the  $\alpha$ -factor pre-pro-leader sequence with the R2 variant is as follows:

*MF $\alpha$ 1* leader sequence-GAAGCTTTG CCG AGT ..3'  
*Hind*III leu pro ser

Leucine is the starting amino acid of mature actinidin polypeptide.

Cloning of the R2 and R5 was carried out by firstly subcloned them into pUC19 vector. The cloned fragments were then digested from pUC19 using *Hind*III and *Sal*I enzymes and ligated with pYSV9 at the *Hind*III and *Sal*I sites. The scheme of cloning of R2 and R5 variants is depicted in Fig. 2.

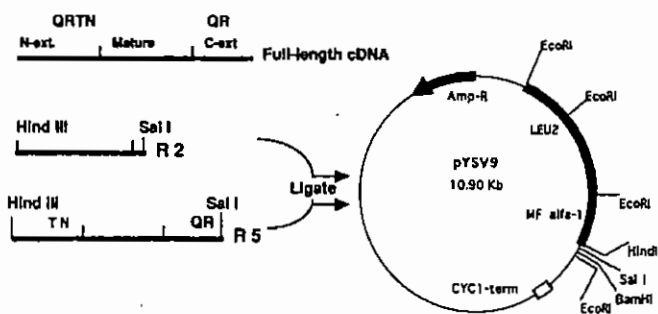


Figure 2. Cloning of actinidin-encoding DNA variant R2 and R5 into a yeast expression-secretion vector, pYSV9. QR is the putative vacuolar targeting signal. MF- $\alpha$ 1 is the pre-leader sequence of *MF $\alpha$ 1* gene of *S. cerevisiae* used to drive the expression of actinidin-encoding gene and direct the polypeptide to the secretion pathway. CYC1-term is the transcription terminator derived from CYC1 gene.

The recombinant plasmid pYSV-R2 and pYSV-R5 obtained were then used for transformation of *S. cerevisiae* strain DBY 746. Transformants were subsequently grown in 200 ml of YEPD for 24 hours. Supernatant was then chromatographed through DEAE Sephacel column. Selected peaks of protein were electrophoresed on an SDS-PAGE followed by immunoblotting on a nitrocellulose membrane. Figure 3 shows the elution profile of supernatant obtained from the cultures of *S. cerevisiae* harboring recombinant actinidin gene construction

pYSV-R2 (Fig. 3A), pYSV-R5 (Fig. 3B), and a control culture carrying pYSV9 (Fig. 3C).

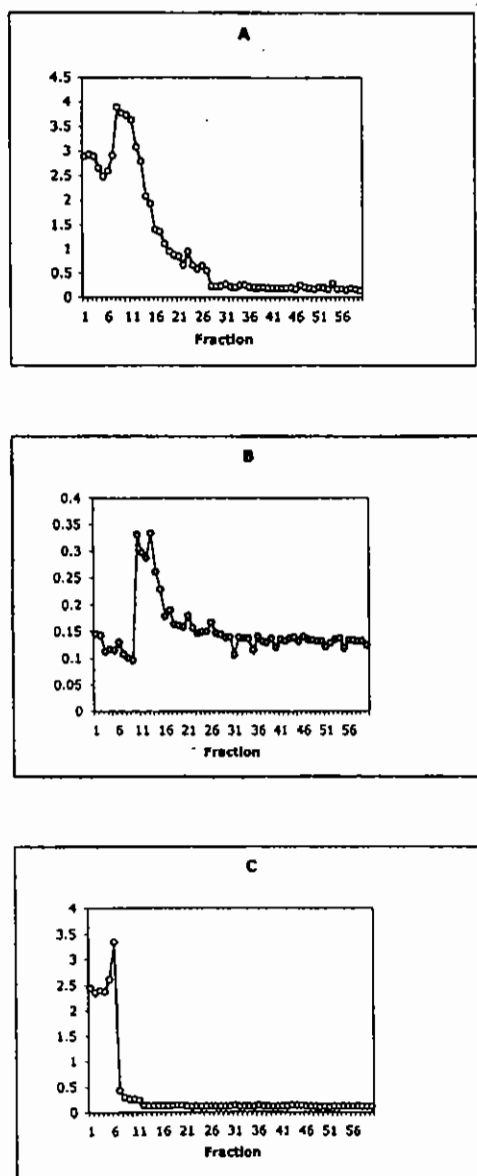


Figure 3. Elution profile of supernatant protein of yeast harboring different actinidin gene constructions cultivated in 200 ml of complete medium (YEPD). A: yeast carrying pYSV9-R2, B: yeast carrying pYSV9-R5, C: yeast carrying pYSV9 (plasmid vector without any foreign gene insert).

The results of electrophoresis and immunoblotting of selected peaks of protein is depicted in Fig. 4. Fraction numbers of protein electrophoresed were: 8 and 23 (for pYSV-R2), 10 and 13 (for pYSV-R5), and 6

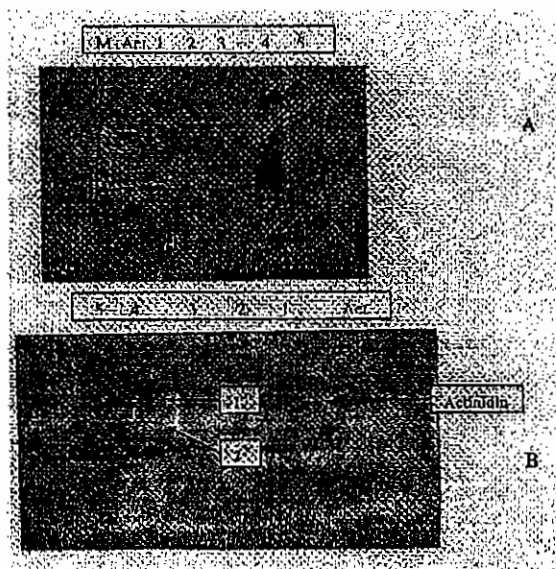
(for pYSV9). As a control for immunoblotting analysis, a purified actinidin was also loaded on the gel.

The results of electrophoresis and immunoblotting demonstrated that samples of supernatant from the culture harboring pYSV-R2 reacted immunogenically with the anti-actinidin antibody (Fig. 4, lane 4, see arrows). On the other hand, samples of supernatant from the culture harboring pYSV-R5 and pYSV9 did not show a significant specific immunogenic reaction with the anti-actinidin antibody, despite the fact that several non-specific bands appeared. Non-specific reaction was also observed even in the samples of supernatant from pYSV-R2. Similarly, when another actinidin-encoding gene construction was expressed in yeast, the intracellular preparation of the cell also demonstrated non-specific reaction with the antibody (Yuwono, 1991). This non-specific reaction may be attributed to the fact that the first antibody used was of polyclonal that might also react with intracellular proteins of yeast. The appearance of protein bands (Fig. 4, lane 2, see arrow no.1) reacted specifically with the antibody suggested that they resemble native actinidin, despite the fact that the size was slightly bigger than the native actinidin (lane Act). A smaller protein band that also reacted specifically with the antibody (arrow no. 2) may represent a degraded recombinant actinidin molecule.

The size of protein band in Fig. 4 (lane 2, arrow no. 1) is slightly bigger than the native actinidin. Previously, it was also found that a variant of actinidin-encoding DNA consisting of mature actinidin sequences plus the whole C-terminal extension was expressed in yeast but resulted in a slightly bigger protein product. The protein, however, was retained inside the cell and not secreted. On the other hand, the full-length actinidin cDNA, consisting of the whole N-terminal extension, mature actinidin



sequences, and the whole C-terminal sequences, was expressed and resulted in a protein band of equal size to that of native actinidin but was not secreted from the cell (Yuwono, 1991). These observations, including the current work, suggest that the N-terminal extension and C-terminal extension are required in the processing of actinidin precursor following translation. In this work, it was observed that variant of actinidin-encoding DNA that did not include the N-terminal extension but still retained part of the C-terminal extension (with no QR motif) was expressed and the protein product was secreted into the culture supernatant. This DNA variant (pYSV9-R2) resulted in an incompletely processed actinidin precursor which, therefore, had a bigger size than the native actinidin.



**Figure 4.** Results of electrophoresis (A) and immunoblotting (B) of supernatant protein of yeast cultures harboring actinidin gene constructions pYSV9-R2 and pYSV9-R5 cultivated in a complete medium (YEPD). 1: supernatant of culture carrying plasmid vector only (pYSV9), 2: supernatant of culture carrying pYSV9-R5 fraction no. 10, 3: supernatant of culture carrying pYSV9-R5 fraction no. 13, 4: supernatant of culture carrying pYSV9-R2 fraction no. 8, and 5: supernatant of culture carrying pYSV9-R2 fraction no. 23. Arrows (1 and 2) showed protein bands reacted immunospecifically with the anti-actinidin antibody.

The second DNA variant (pYSV9-R5) did not result in the expression of a protein in the supernatant which reacted immunogenically with the anti-actinidin antibody. The failure to detect a protein product in the supernatant may be attributed to the fact that this variant still retains the QR motif in the C-terminal extension. It was assumed that the QR motif is a putative vacuolar translocation signal (Yuwono, 1991). Therefore, the presence of such motif in the pYSV9-R5 variant might have prevented the polypeptide from being secreted as it might have been diverted into vacuolar compartment.

The pattern of expression of actinidin-encoding DNA variants as described in this work has provided more support to previous study (Yuwono, 1991) that the N-terminal extension and C-terminal extension of the actinidin precursor are required for the correct processing of actinidin. It was also observed that the QR motif may also function as an intracellular (presumably vacuole) translocation signal. Studies have demonstrated that specific organellar translocation signals are present in the sequences of several proteins. For example, the motif Q R was found in the pro-region of vacuolar carboxypeptidase Y of yeast (Johnson *et al.*, 1987; Valls *et al.*, 1987). Similarly, such sequence was also found in the pro-region of other plant vacuolar proteins: phaseolin (QRFD), pea legumin (QRDE), ricin (QRDG), phytohemagglutinin (QRDA), suggesting that the QR is the most conserved motif (Tague *et al.*, 1990). Further study is, therefore, required to establish the exact role of the QR(TN) motif in the translocation of actinidin.

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## References

- Baker, E. N. 1980. Structure of actinidin, after refinement at 1.7 Å resolution. *J. Mol. Biol.* 141: 441-484.
- Brake, A.J., Merryweather, J.P., Coit, D.G., Heberlein, U.A., Masiarz, F.R., Mullenbach, G.T., Urdea, M.S., Valenzuela, P., and Barr, P.J. 1984.  $\alpha$ -factor directed synthesis and secretion of mature foreign proteins in *Saccharomyces cerevisiae*. *Proc.Natl.Acad.Sci. USA* 81:4642-4646.
- Carne, A. and Moore, C. H. 1978. The amino acid sequence of the tryptic peptides from actinidin, a proteolytic enzyme from the fruit of *Actinidia chinensis*. *Biochem. J.* 173: 73-83.
- Hames, B. D and Rickwood, D. 1990. *Gel Electrophoresis of Proteins. A Practical Approach*. Second edition. IRL Press, Oxford.
- Johnson, L. M., Bankaitis, V. A. and Emr, S.D. 1987. Distinct sequence determinants direct intracellular sorting and modification of a yeast vacuolar protease. *Cell* 48: 875-885.
- McDowall, M. A. (1970). Anionic proteinase from *Actinidia chinensis*. Preparation and properties of the crystalline enzyme. *Eur.J.Biochem.* 14: 214-221.
- Paul, W., Amiss, J., Try, R., Praekelt, U., Scott, R., and Smith, H. 1995. Correct processing of the kiwifruit protease actinidin in transgenic tobacco requires the presence of the C-terminal propeptide. *Plant Physiol.* 108: 261-268.
- Podivinsky, E., Forster, R.L.S., and Gardner, R.C. 1989. Nucleotide sequence of actinidin, a kiwi fruit protease. *Nucleic Acid Res.* 17:8363.
- Praekelt, U. M. 1987. Molecular Analysis of Actinidin. PhD thesis. University of Leicester.
- Praekelt, U. M., McKee, R. A. and Smith, H. (1988). Molecular analysis of actinidin, the cysteine proteinase of *Actinidia chinensis*. *Plant. Mol. Biol.* 10: 193-202.
- Rose, M., Winston, F. and Hieter, P. 1990. *Methods in Yeast Genetics*. Cold Spring Harbor Laboratory Press.
- Sambrook, J., Fritsch, E. F. and Maniatis, T. 1989. *Molecular Cloning. A Laboratory Manual*. Second edition. Cold Spring Harbor Laboratory Press.
- Snowden, K. C. and Gardner, R.C. 1990. Nucleotide sequence of an actinidin genomic clone. *Nucleic Acid Res.* 18: 6684.
- Tague, B.W., and Chrispeels, M.J. 1987. The plant vacuolar protein, phytohemagglutinin, is transported to the vacuola of transgenic yeast. *J. Cell Biol.* 105:1971-1979.
- Valls, L.A., Hunter, C.P., Rothman, J.H., and Stevens, T.H. 1987. Protein sorting in yeast: the localization determinant of yeast vacuolar carboxypeptidase Y resides in the propeptide. *Cell* 48:887-897.
- Yuwono, T. 1991. A Study of Actinidin Expression in Yeast. PhD thesis. University of Leicester, UK.